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USE OF MAGNETIC BEAD RESIN AND AUTOMATED LIQUID HANDLER EXTRACTION METHODS TO ROBOTICALLY ISOLATE NUCLEIC ACIDS OF BIOLOGICAL AGENT SIMULATES

Tom C.M. Seegar
Peter A. Emanuel

CHEMICAL BIOLOGICAL SERVICES DIRECTORATE

Andrew M. Bailey
Isaac R. Fruchey

Battelle

BATTELLE MEMORIAL INSTITUTE
Aberdeen, MD 21001

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Aberdeen Proving Ground, MD 21010-5424

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The events that occurred following the mailing of *Bacillus anthracis*-laced envelopes through the postal system highlight the need to perform biological screening on large numbers of environmental samples. High-throughput screening that relies on integrated robotic systems to speed analysis has been undertaken to handle the surge in samples requiring testing in events involving weapons of mass destruction. These automated screening systems require DNA extraction methods capable of handling environmental samples that contain inhibitors and have target organisms at low concentrations. This study describes the development of a method for testing paramagnetic bead-based resin for use with an automated liquid handler and environmental samples. The DNA purification kits for food and blood were capable of purifying DNA from bacteria used to simulate biological weapons. An optimized hybrid protocol combining components from these two kits gave the best purification results, as determined by real time-polymerase chain reaction. Using this protocol, the gram-negative bacteria *Erwinia herbicola* could be detected as low as 1 CFU/ml starting concentration, and *Bacillus subtilis* var. *niger* spores were detectable at 10,000 CFU/ml. When combined with bead beating, these spores were consistently detectable at 100 CFU/ml. Furthermore, the automated approach significantly shortens analysis time versus manual operations, and has the capacity to purify nucleic acid from a variety of environmental samples.

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PCR – Polymerase chain reaction
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PREFACE

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USE OF MAGNETIC BEAD RESIN AND AUTOMATED LIQUID HANDLER EXTRACTION METHODS TO ROBOTICALLY ISOLATE NUCLEIC ACIDS OF BIOLOGICAL AGENT SIMULATES

1. INTRODUCTION

Events in the United States during 2001 have renewed interest in routine biological monitoring of the environment for biological warfare agents. As previously predicted, public health laboratories were ill-prepared to deal with a large influx of samples during a national incident, and a high-throughput sample screening solution was needed (16). As a model, diagnostic laboratories are faced with high numbers of urgent samples each day in which the results must carry a high level of confidence. Diagnostic samples are presented, however, in preordained sample matrices such as blood, urine, nasal swabs, or feces. Although these matrices can often prove a challenge for sample clean up and nucleic acid isolation, the limited number of matrices allows for standardized approaches in methodology to prepare nucleic acids and remove inhibitors. When samples are taken from the environment and can come from aerosol samplers or surface wipes of unknown samples, there is a wide spectrum of potential inhibitors that must be considered (22). Based on our previous work of extracting DNA from environmental samples, bead-based methods were preferable to avoid organic extractions, centrifugation, or filtration steps that can result in clogging. Magnetic bead resins were advantageous because they allow the option of aggressive washes and fluid-like dynamics that can aid in the removal of particulate matter and potential PCR inhibitors as part of a fully automated system. Magnetic bead-based DNA extraction has been used to successfully isolate nucleic acids from a variety of sample types that contain PCR inhibitors (2, 9, 11, 21). MagneSil™ (Promega, Madison, WI) was selected for study as a paramagnetic bead-based nucleic acid extraction system that offers a variety of porosities and size options for use with food, blood, and forensic samples (19).

The automated liquid handler system selected was required to be versatile and programmable for DNA extraction as well as PCR assay set-up. The full integration of the system was required since the final marriage of magnetic bead and automated liquid handler was only one piece of a larger robotic system for screening large numbers of samples for biological weapons. The Automated Biological Agent Testing System (ABATS) (patent pending), initiated by the Department of Defense in response to homeland defense initiatives, is designed to combine PCR and electrochemoluminescent (ECL) immunoassay to analyze each environmental sample, rather than relying on just one technology or the other (see figure). The Biomek® FX liquid-handling robotic system (Beckman-Coulter, Fullerton, CA) allows the ABATS to use a CORE system to link the Applied Biosystems ABI Prism® 7900HT thermocycler and the OR*IGEN*® M8 Analyzer (IGEN, Gaithersburg, MD) to form an integrated screening laboratory. The overall goal of the ABATS program is a three-fold increase in sample throughput with a two-fold reduction in cost per sample when compared with current manual screening operations.

To realize this goal, manual procedures requiring timesaving measures were sample chain of custody paperwork, nucleic acid extraction, reaction set-up, and result interpretation. In this study, we investigate timesaving measures pertaining to nucleic acid extraction of two accepted biological agent simulants in an effort to reduce labor and speed analysis of unknown environmental samples by addressing bottlenecks in the analysis process.

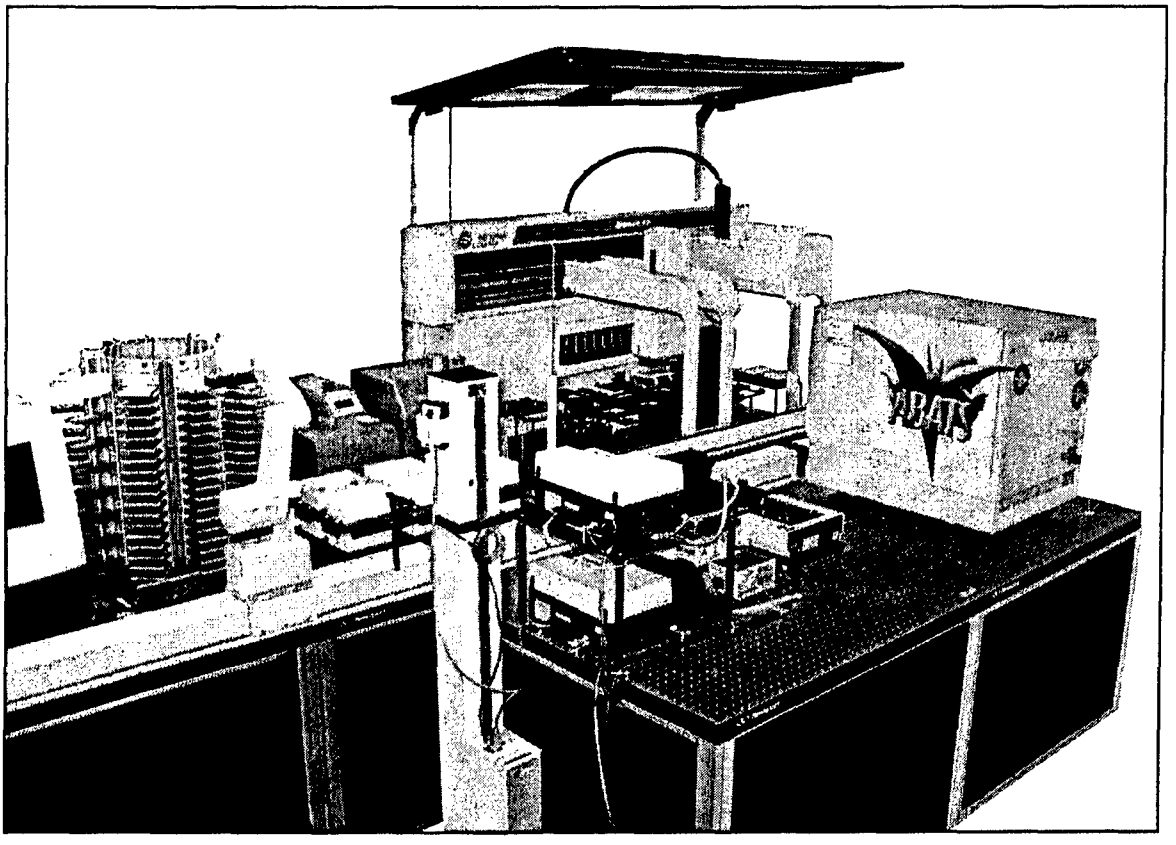

Figure. Automated Biological Agent Testing System (ABATS). The ABATS uses the Biomek® FX liquid handling robotic system to set up immunoassay and PCR reactions, as well as perform DNA extraction.

2. MATERIALS AND METHODS

2.1 Biological Agent Simulants.

Cell suspensions of *Bacillus subtilis* var. *niger* spores (BG spores) and *Erwinia herbicola* vegetative cells were prepared for use as biological simulants. BG spores were prepared by inoculating 1 g spores of *Bacillus subtilis* var. *niger* (Merck & Co., Inc., Whitehouse Station, NJ) in 100 ml irrigation water (McGAW Inc., Irvine, CA). The suspension was mixed with a vortex mixer (Barnstead Thermolyne Type 37600, Bebuque, IA) for 10 min. The mixture was placed in an orbital shaker water bath at 65°C and 50 RPM for 10 min. The suspension was plated on 15 plates of AK Agar #2 Sporulating Agar (Cat. No. 210912, Becton Dickinson, Sparks, MD) using a swab to cover the whole plate. The plates were incubated at 30°C for 7 days. The cells were examined periodically via phase contrast microscopy to examine spore development and formation. The spores were scraped from the plates using sterile swabs and suspended in 400 ml sterile phosphate buffered saline (PBS) (Catalog No. 1000-3, Sigma, St. Louis, MO) and were washed three times with PBS. A titer was established by plating serial dilutions of the washed spore suspension on Tryptose Agar media (Cat. No. 264100, DIFCO, Detroit, MI). The final solution was adjusted to contain $1x10^6$ CFU/ml in PBS. Aliquots of 1 ml were frozen at –20°C until needed. *E. herbicola* suspensions were prepared by inoculating 1 liter of sterile Trypticase Soy Broth (Cat. No. 211771, Becton Dickinson, Sparks, MD) with *E. herbicola* (ATCC No 33243). The culture was incubated at 25°C in a Model G25 orbital shaker (New Brunswick Scientific, Edison, NJ) at 200 RPM for 48 hr. The cells were harvested by low speed centrifugation and washed three times in sterile PBS at 4°C. Serial dilutions of the cells on Trypticase Soy Agar media were plated to determine the titer of the cells. The harvested cells were adjusted to $1x10^6$ CFU/ml with PBS, and 1 ml aliquots were flash frozen at –20°C until needed.

2.2 DNA Isolation.

All DNA extraction procedures were carried out on a Biomek® FX (Beckman Coulter, Fullerton, CA). DNA purification kits for food (Cat. No. FF3750) and blood (Cat. No. MD1360) were kindly provided by Promega (Madison, WI). For each extraction sample, either the BG spore suspension or *E. herbicola* cell suspension was aliquoted into one well of a 96-well 2-ml deep-well plate (Costar 3960). After filling the wells, the deep-well plate was placed onto the deck of the Biomek® FX, where all subsequent pipetting was performed robotically. Also placed onto the deck of the Biomek® FX in deep-well plates were all reagents including isopropanol, 70% ethanol, water, and MagneSil™ Yellow, and either the blood kit or food kit lysis buffers. Following DNA extraction using the Food Kit Protocol, Blood Kit Protocol, or Hybrid Protocol, a shallow 96-well plate containing eluted DNA were frozen at -20°C or used immediately for PCR analysis.

2.3 Food Kit Protocol for Biomek® FX.

Extraction using the food kit was performed as follows: 250 µl of a sample was added to 500 µl Lysis Buffer A and shaken at 1400 RPM for 1 min., 250 ml of Lysis Buffer B was added and shaken at 1300 RPM for 1 min., followed by incubation at room temperature for 10 min. MagneSil™ Yellow (60 µl) was added and shaken at 1200 RPM for 1 min., followed by the addition of 248 µl isopropanol and shaken at 1100 RPM for 5 min. The MagneSil™ particles were removed from the solution in two half-volume steps by 1 min. incubations on a deep-well MagnaBot® (Promega V3031, V8581). The MagneSil™ particles were washed with an additional 250 µl of Lysis Buffer B, shaken at 1500 RPM for 1 min., and recollected with the MagnaBot® (1 min.). The MagneSil™ particles were washed with 70% ethanol (200 µl 70% ethanol, shaken at 1500 RPM for 1 min., recollected with MagnaBot® for 1 min.) three times. Particles were dried for 13 min. at room temperature. The MagneSil™ particles were combined with 100 µl water, incubated at room temperature for 1 min., shaken at 1500 RPM for 1 min., and the MagneSil™ was removed using the MagnaBot® (1 min.). The eluted DNA in water was transferred into a shallow 96-well plate.

2.4 Blood Kit Protocol for Biomek® FX.

Extraction using the blood kit was performed as follows: 200 µl of sample was combined with 50 µl of MagneSil™ and 310 µl of Blood Kit Lysis Buffer and shaken at 900 RPM for 1 min. followed by incubation at room temperature for 6 min. MagneSil™ particles were collected using two successive MagnaBot® incubations (1 min. and 30 sec.) with effluent discarded between each incubation. Additional Blood Kit Lysis Buffer (360 µl) was added to the MagneSil™ particles, shaken at 1250 RPM for 1 min., and the particles were again collected in two successive MagnaBot® incubations (1 min. and 30 sec.). The MagneSil™ particles were washed using a salt wash solution [add 180 µl Salt Wash, shake at 1250 RPM for 1 min., incubate at room temperature for 1 min., add 180 µl Salt Wash, shake at 1250 RPM for 1 min., incubate at room temperature for 1 min., incubate on MagnaBot® (1 min.), remove effluent, incubate on MagnaBot® (30 sec.), remove all remaining effluent], and repeat twice. MagneSil™ particles were then washed using an alcohol wash solution [add 180 µl 2X Alcohol Wash, shake at 1250 RPM for 1 min., incubate at room temperature for 1 min., add 180 µl 2X Alcohol Wash, shake at 1250 RPM for 1 min., incubate at room temperature for 1 min., incubate on MagnaBot® (1 min.), remove effluent, incubate on MagnaBot® (30 sec.), remove all remaining effluent] and repeat three times. MagneSil™ was then dried for 15 min. at room temperature, and purified DNA was eluted by the addition of 210 µl elution buffer, shaken at 1300 RPM for 1 min., incubated at room temperature for 5 min., and removed MagneSil™ particles with a 1 min. incubation on the MagnaBot®. Effluent (200 ml) was drawn off and transferred to a shallow 96-well plate.

2.5 Hybrid Protocol for Biomek® FX.

The Hybrid Protocol was performed as follows: 625 µl of a sample was added to 625 µl Blood Kit Lysis Buffer and shaken at 900 RPM for 1 min., followed by incubation at room temperature for 10 min. MagneSil™ Yellow (60 µl) was added and shaken at 900 RPM for 1 min., followed by the addition of 250 µl isopropanol and mixing by pipetting, on the Biomek, for 5 min. The MagneSil™ particles were removed from the solution in two half-volume steps by 1 min. incubations using the MagnaBot®. The MagneSil™ particles were washed with an additional 250 µl of Blood Kit Lysis Buffer, shaken at 1500 RPM for 1 min., and recovered with the MagnaBot® for 1 min. The MagneSil™ particles were washed with 70% ethanol (200 µl 70% ethanol, shaken at 1500 RPM for 1 min., recovered with MagnaBot® for 1 min.) three times. Particles were dried for 13 min. at room temperature. Purified DNA was eluted into water (100 µl water, incubated at room temperature for 1 min., shaken at 1500 RPM for 1 min., heated at 65°C for 3 min., shaken at 1500 RPM for 1 min., and MagnaBot® for 1 min.) and transferred into a shallow 96-well plate.

2.6 Physical Disruption of BG Spores.

A dilution series of BG spores and *E. herbicola* were combined in a 2 ml screw cap microfuge tube with 1 ml cell solution, 0.16 g of 0.1 mm glass beads, and 0.03 g of 2 mm zirconium beads (Cat. No. 11079110, 11079124zx, BioSpec Products, Inc., Bartlesville, OK). Samples were processed in a Mini-BeadBeater-8 (BioSpec Products, Inc.) for 30 min. in PBS+T (0.3% $Tween_{20}$; Cat. No. P-9416, Sigma, St. Louis, MO) at 3200 RPM. Processed samples were removed and pelleted in a microcentrifuge at 12,000 x g for 2 min. The supernatant was removed, and 625 µl was processed in the hybrid method as described.

2.7 Manual DNA Extraction.

Manual DNA extraction followed the same protocol as the optimized automated extraction, with the exception that all pipetting steps were performed manually with a single-channel and an eight-channel pipette.

2.8 Polymerase Chain Reaction.

Amplification, data acquisition, and data analysis was carried out on an Applied Biosystems model 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Primer and probe sequences for BG detection were developed at the U.S. Army Edgewood Chemical Biological Center (ECBC), APG, MD. Primer and probe sequences for *E. herbicola* detection were developed by the Naval Medical Research Center (Silver Spring, MD). PCR reactions were performed in 20 µl volumes in 384-well microtiter plates. Each reaction was set up using Taqman® Universal Master Mix (4304437, Applied Biosystems, Foster City, CA), 500 nM forward and reverse primer, 200 nM FAM/TAMRA labeled fluorogenic probe, and 8.5 µl of extracted DNA product per reaction. The thermocycler conditions were as follows: 50°C for 2 min., 95°C for 10 min., and 50 cycles of 95°C for 15 sec., and 60°C for 1 min. Analysis was performed using Sequence Detection Software v.2.0.

2.9 Environmental Sample Analysis.

Surface swipes were collected using a Biological Sampling Kit (BiSkit) (ECBC, APG, MD), which dispensed the sample in approximately 4 ml phosphate buffered saline, pH 7.4 plus 0.3% Tween-20 (PBS+T). Air samples were collected in metropolitan areas using a Dry Filter Unit (DFU) 1000 (Joint Program Executive Office for Chemical and Biological Defense, Department of Defense) for 12 hr at a flow rate of 300-600 l/min. Filters were removed and placed into 10 ml of sterile PBST, and vortexed for 1 min. to generate filter washes. Six soil samples provided by the Soil Society of America's North American Proficiency Testing Program (Alabama, Big Horn, Brookings, Keenesburg, Teller, and Whitewater) and two uncharacterized soil samples (Baghdad and Kahmasia) were mixed with PBST at the ratio of 1 ml of PBST per 0.5 g soil. Samples were mixed by inversion for 10 min. at room temperature. Soil particles were removed by centrifugation, and the supernatant was designated as soil washes. The inherent presence of PCR inhibitors was determined by using soil washes, air filter washes, and surface swipe samples as described above and adding them directly into a PCR reaction for *E. herbicola* at 1/10 of the final volume. PCR was run as described, and the change in C_T value was compared with the C_T value of the same reaction without environmental matrix added. Serial 10-fold dilutions were created and tested likewise until a non-inhibitory dilution ($\Delta CT = 3 \times StDev$) was found for each sample matrix. From the filter washes, surface samples, and soil washes, 620 µl were removed and added to 5 µl of 1.25×10^8 CFU/ml of *E. herbicola* suspensions to generate a final concentration of 1×10^6 CFU/ml. The 625 µl of spiked washes were used for DNA extraction using the hybrid protocol. For BiSKit samples and soil washes that showed inhibition following DNA extraction, dilutions were generated at the ratios 1:5 and 1:25 for BiSKit samples, and 0.5 g/5 ml and 0.5 g/25 ml for soil washes, and retested.

2.10 Filter Induced Sampling.

The filters for this study were 47 mm in diameter polyester felt disk made by the American Felt and Filter Company (New York, NY). Different dilutions of *B. subtilis* and *E. herbicola* were prepared from a stock of 1.8×10^9 CFU/ml. The filters were spiked with 1 ml portions and allowed to dry overnight in a ventilation hood. The test samples were prepared in 50 ml screw cap vials by adding through the filters inside the vial, 10 ml of PBS+T (0.3% $Tween_{20}$; Cat. No. P-9416, Sigma, St. Louis, MO). The vials intended for filter extraction with beads also had an addition of 5 ml of 2 mm zirconium beads (Cat. No. 11079124zx, BioSpec Products, Inc., Bartlesville, OK). The samples were hand shaken for 2 min. After shaking, 1 ml was extracted and placed in a 2 ml screw top vial with 0.16 g of 0.1 mm glass beads (Cat. No. 11079110, BioSpec Products, Inc., Bartlesville, OK) and homogenized with a BioSpec Mini Bead-Beater 8 for 30 min. After homogenization, the samples were centrifuged at 12000 RPM for 2 min. with an Eppendorf Centrifuge 5415D. Then 625 µl of the samples were aliquoted into a 96-well 2-ml deep-well plate (Costar 3960) and placed onto the deck of the Biomek® FX where the automated DNA extraction procedure was performed.

3. RESULTS

3.1 Biomek® FX DNA Extraction.

Promega MagneSil™ DNA purification resin with a macroporous composition is commercially available for use with food and blood, and these kits were used as a starting point to develop a purification method for environmental samples to extract DNA from *E. herbicola* vegetative cells and from *Bacillus subtilis* var. *niger* (BG) spores. Real-time PCR was used to evaluate the effectiveness of the nucleic acid extraction for the two organisms as the total quantities of DNA isolated were low, and the overall objective was to more efficiently integrate the extraction process with PCR analysis and increase the level of detection using PCR (5). TaqMan® polymerase chain reaction is a probe hydrolysis method that measures the progress of PCR amplification by measuring light in a technique called fluorescence resonance energy transfer (FRET) (6, 12). Researchers from the U.S. Army and U.S. Navy provided the TaqMan® PCR primer and probe sequences that specifically amplify genes within *Erwinia* and BG. The Applied Biosystems model 7900HT thermocycler is a TaqMan® based instrument in which the interpretation of a positive response is measured when a reaction breaches a threshold (C_T) when referenced against a non-responsive baseline. Thresholds that occur at earlier cycles for the same PCR assay can be correlated with a higher starting concentration of template DNA in the sample. Thus, a sample that breaches the threshold at cycle 30 can be inferred to have contained a higher amount of starting DNA than a sample that crosses the threshold at cycle 42. Using the unmodified food kit, average C_T scores of 29 and 40 were obtained for *E. herbicola* (1×10^5 CFU/ml) and BG (1×10^6 CFU/ml), respectively (Table 1). Using the unmodified blood kit, C_T scores of 27 and 44 were observed. An optimized hybrid protocol of these two kits that used the method from the food kit and the chaotropic lysis buffer from the blood kit proved to be the better combination, resulting in improved C_T values of 26 and 36, respectively. The blood lysis buffer has a more concentrated formulation of guanidinium salts than is found in the food kit (personal communication, Promega Corporation), and that component may facilitate more efficient lysis or recovery of DNA from environmental samples.

3.2 Detection Limits.

Using the optimized hybrid protocol, it was determined that the detection limit for *E. herbicola* vegetative cells was between 1 and 10 CFU/ml (Table 2). Detection was not observed with a sample of 0.1 CFU/ml (data not shown). BG spores were detected at 100,000 CFU/ml in 91.7% of samples (11/12), and at 10,000 CFU/ml in 8.3% of samples (1/12) using the hybrid protocol. For both BG and *E. herbicola*, the C_T values increased by approximately 3.3 for each 10-fold dilution, which is indicative of optimal thermocycling efficiency under the conditions tested (Applied Biosystems, User Bulletin #2: ABI Prism 7700 Sequence Detection System, December 11, 1997).

In an effort to drive detection thresholds even lower for spore containing samples, additional pre-treatment lysis methods were tested to augment the hybrid extraction protocol. Boiling the cell suspensions for 30 min. prior to extraction was tested in an effort to disrupt the tough exosporium that surrounds *Bacillus* species of bacteria. This process did not significantly

contribute to enhanced PCR detection (data not shown). Bead beating BG spore suspensions prior to performing the hybrid method was successful in increasing the detection limit of BG down to 1 x 10^2 CFU/ml 91% of the time, and had no deleterious affect on the purification of *E. herbicola* DNA. These results suggest that the hybrid protocol with no bead beating pretreatment is likely purifying residual DNA that remains trapped on the exosporium of the BG spore, whereas bead beating probably disrupts the BG spores.

3.3 Manual Extraction.

To compare the sensitivity and reliability of the automated procedure, the hybrid process was performed manually on the same samples using an eight-channel multi-pipettor and testing by PCR. The manual extraction results were very similar to the results obtained with the Biomek® FX with respect to C_T scores, although the manual extraction was less consistent in detecting both bacteria (Table 2). Manual extraction was less effective than automated extraction with BG spores, with a reduced detection limit of 1 x 10^5 CFU/ml for 17% of the samples. Furthermore, performing the hybrid extraction protocol manually took approximately 150 min., whereas the automated Biomek® FX protocol took only 70 min.

3.4 Environmental Sampling.

Washes from air filters, soil, and surface wipes were generated to test the effectiveness of the MagneSil® Hybrid protocol and its ability to remove potential PCR inhibitors commonly associated with environmental samples. The initial inhibition present within each sample was gauged by changes in the threshold (C_T) when the samples were tested by *E. herbicola* PCR and compared to the threshold observed with buffer alone. Each sample was diluted systematically until it no longer inhibited PCR. Dilutions ranged from 1:10,000 to 1:10,000,000, with a higher dilution factor indicating a higher concentration of inhibitors within the starting sample (Table 3). To examine the hybrid protocol's ability to clean the environmental samples of these inhibitors, the environmental washes were spiked with 1 x 10^6 CFU/ml of *E. herbicola* cell suspension, and the DNA extraction protocol was performed in the same manner as the previous experiment. No significant PCR inhibition was observed in undiluted air filter washes, three surface swipe samples, and one soil type (Table 3). Most soil washes and two swipe samples had to be diluted (resuspended in a larger volume of buffer) to show minimal inhibition, although for this study, the samples containing the most inhibitors (Bighorn soil and mailbox surface swipe) showed at least a 2000-fold removal of PCR inhibitors using the hybrid method. It is apparent that the hybrid protocol was effective in removing the vast majority of PCR inhibitors from all samples. We plan to test a dilution of each sample or use an internal positive control to test for inhibition when evaluating environmental samples.

3.5 Filter Induced Analysis.

To compare the limited detection concentrations of the spiked filters, the different concentrations were made and placed on the filters and analyzed on the Biomek® FX so that the C_T scores could be compared (Table 4). The *B. subtilis* samples were detected in concentrations as low as $1.0x10^5$ CFU/ml with the *B. subtilis* detector. The *E. herbicola* samples were detected in lower concentrations of $1.0x10^3$ CFU/ml with the *E. herbicola* detector when compared to the *B. subtilis* samples. As expected, the *E. herbicola* was not detected with the *B. subtilis* detector, and the *B. subtilis* was not detected with the *E. herbicola* detector, except in the sample *B. subtilis* with a concentration of $1x10^6$ CFU/ml with 2 mm zirconium beads. The reliability of this sample was below 99% with a reliability of 66.7% (data not shown). The samples with both simulants spiked in the filters showed C_T scores that resemble the C_T scores of nonmixed samples of the same concentration.

To compare the effect of filter extraction in the presence of beads on the samples, a subsequent test was performed where the samples were shaken with beads and analyzed on the Biomek® FX so that the C_T scores could be compared (Table 4). The C_T scores for both *B. subtilis* and *E. herbicola* do not prove to be beneficial in lowering the C_T scores of the samples with beads when compared with samples without beads.

Table 1: Threshold of detection (C_T) for the purification of *Bacillus subtilis* var. *niger* and *Erwinia herbicola* DNA using Promega Blood Method, Promega Food Method, and Hybrid Method.

	C_T Values		
	Blood Method	*Food Method*	*Hybrid Method*
B. subtilis[a]	43.7 ± 2.2	40.3 ± 0.8	36.2 ± 1.2
E. herbicola[b]	27.3 ± 0.2	28.6 ± 0.5	25.6 ± 0.3

[a] Starting concentration of $1x10^6$ CFU/ml, assays performed using 10 replicates.
[b] Starting concentration of $1x10^5$ CFU/ml, assays performed using 10 replicates.

Table 2: Comparison of *Bacillus subtilis* var. *niger* and *Erwinia herbicola* extraction using a Biomek® FX, manual pipetting steps, and physical disruption prior to Biomek® FX extraction

Sample Concentration (CFU/ml)	Biomek® FX Hybrid Extraction (% Detected)		Manual Hybrid Extraction (% Detected)		Biomek® FX Hybrid Extraction with Beadbeating (% Detected)	
	B. subtilis	*E. herbicola*	*B. subtilis*	*E. herbicola*	*B. subtilis*	*E. herbicola*
1×10^6	100	100	91.7	91.7	100	100
1×10^5	91.7	100	16.7	100	100	100
1×10^4	8.3	100	0	100	100	100
1×10^3	0	100	0	100	100	100
1×10^2	0	100	0	100	91.7	91.7
1×10^1	0	100	0	83.3	0	100
Water	0	0	0	0	0	0

Percentages calculated from 12 samples at each dilution.

Table 3: Detection of *Erwinia herbicola* in environmental samples using the hybrid purification method and real-time PCR analysis

Environmental Samples		PCR Inhibition [a]	Minimal Variance in Threshold ($\Delta C_T <= 2$)
Soils [b]			
	Alabama	++++	0.5 g soil / 1 ml buffer
	Baghdad	++++	0.5 g soil / 25 ml buffer
	Big Horn	+	0.5 g soil / 5 ml buffer
	Brookings	++	0.5 g soil / 5 ml buffer
	Kahmasie	++	0.5 g soil / 25 ml buffer
	Keenesburg	++++	0.5 g soil / 25 ml buffer
	Teller	+++	0.5 g soil / 5 ml buffer
	Whitewater	+++	0.5 g soil / 5 ml buffer
Air Filters [c]		++++	1 Filter / 10 ml buffer
Surface Swipes [d]			
	Desk	+	No dilution needed
	Front Door	++++	No dilution needed
	HVAC Duct	++++	1:25 dilution needed
	Mailbox	+	1:5 dilution needed
	Vending Machine	+++	No dilution needed

[a] ++++ = $1:10^7$ Dilution is required to remove inhibition, +++ = $1:10^6$ Dilution is required to remove inhibition, ++ = $1:10^5$ Dilution is required to remove inhibition, + = $1:10^4$ Dilution is required to remove inhibition

[b] Soil samples were analyzed in triplicate for each soil type

[c] Air filter washes are an average of 10 independently collected filters

[d] Surface swipes were collected with a BiSkit surface sampler and assayed in triplicate

Table 4: Comparison of mean C_T values for different concentrations in filters of *Bacillus subtilis* var. *niger* and *Erwinia herbicola* using bead-aided filter extraction and nonbead-aided filter extraction prior to Biomek® FX extraction and real-time PCR analysis

Sample Name and Concentration	Detector	Mean Ct	Detector	Mean Ct
E. herbicola $1x10^6$	*B. subtilis*	50.00	*E. herbicola*	27.29
E. herbicola $1x10^6$ with beads	*B. subtilis*	50.00	*E. herbicola*	27.19
E. herbicola $1x10^5$	*B. subtilis*	50.00	*E. herbicola*	30.41
E. herbicola $1x10^5$ with beads	*B. subtilis*	50.00	*E. herbicola*	31.03
E. herbicola $1x10^4$	*B. subtilis*	50.00	*E. herbicola*	33.60
E. herbicola $1x10^4$ with beads	*B. subtilis*	50.00	*E. herbicola*	35.22
E. herbicola $1x10^3$	*B. subtilis*	50.00	*E. herbicola*	36.94
E. herbicola $1x10^3$ with beads	*B. subtilis*	50.00	*E. herbicola*	39.94
E. herbicola $1x10^2$	*B. subtilis*	50.00	*E. herbicola*	50.00
E. herbicola $1x10^2$ with beads	*B. subtilis*	50.00	*E. herbicola*	50.00
E. herbicola $1x10^1$	*B. subtilis*	50.00	*E. herbicola*	50.00
E. herbicola $1x10^1$ with beads	*B. subtilis*	50.00	*E. herbicola*	50.00
B. subtilis $1x10^6$	*B. subtilis*	38.89	*E. herbicola*	50.00
B. subtilis $1x10^6$ with beads	*B. subtilis*	37.63	*E. herbicola*	35.59
B. subtilis $1x10^5$	*B. subtilis*	36.59	*E. herbicola*	50.00
B. subtilis $1x10^5$ with beads	*B. subtilis*	46.49	*E. herbicola*	50.00
B. subtilis $1x10^4$	*B. subtilis*	50.00	*E. herbicola*	50.00
B. subtilis $1x10^4$ with beads	*B. subtilis*	50.00	*E. herbicola*	50.00
B. subtilis $1x10^3$	*B. subtilis*	50.00	*E. herbicola*	50.00
B. subtilis $1x10^3$ with beads	*B. subtilis*	50.00	*E. herbicola*	50.00
B. subtilis $1x10^2$	*B. subtilis*	50.00	*E. herbicola*	50.00
B. subtilis $1x10^2$ with beads	*B. subtilis*	50.00	*E. herbicola*	50.00
B. subtilis $1x10^6$ & *E. herbicola* $1x10^7$ with beads	*B. subtilis*	40.65	*E. herbicola*	26.85
B. subtilis $1x10^6$ & E. *herbicola* $1x10^7$	*B. subtilis*	39.12	*E. herbicola*	27.33
B. subtilis positive control	*B. subtilis*	32.01	-	-
E. herbicola positive control	-	-	*E. herbicola*	24.25

4. DISCUSSION

Through the combination of two magnetic bead-based DNA purification kits, an effective automated protocol was created for the purification of nucleic acids from spore-forming and vegetative bacteria, by using biological warfare agent simulants *Bacillus subtilis* var. *niger* and *Erwinia herbicola*. The military often employs biological simulants to represent highly pathogenic biological weapons and to perform research and testing functions in a safe and cost effective manner. *Erwinia herbicola* is a biological simulant for gram-negative vegetative bacteria such as *Yersinia pestis*, and *Bacillus subtilis* var. *niger* spores, also called BG spores, are a gram positive spore forming bacterial simulant meant to mimic *Bacillus anthracis* (4). Although detection of microorganisms by real time-PCR can be accomplished without a separate DNA purification step for some types of samples (8, 10), the removal of PCR inhibitors from dirty environmental samples necessitates DNA purification techniques. Paramagnetic bead-based DNA purification has proven effective in generating "clean" PCR template, and has been adapted for automated platforms developed to screen samples by the U.S. military. Bead-based

methods allow fluid-like dynamics, and the beads can be fully resuspended during washing steps to facilitate efficient removal of potential inhibitors (19, 20, 21).

The hybrid method described here uses a macroporous silica resin that surrounds a ferrous oxide core. This resin was developed for food matrices; but, the hybrid method improved its use for environmental samples by the incorporation of the lysis buffer from the blood kit. The lysis buffer from the blood kit contains a higher concentration of guanidinium chaotrope when compared to the two-part lysis buffer from the food purification kit (personal communication, Promega Corporation). This higher concentration of chaotropic salt may result in improved recovery of nucleic acid material and the reduction in the thermocycling detection threshold (C_T score) when processing biological simulants. An additional advantage is using only one lysis buffer, resulting in a faster processing time and fewer consumables. It is likely that the chaotrope produces more efficient lysing of vegetative cells and more efficient recovery of trapped DNA that is within the spore sample.

The detection limits observed for *E. herbicola* are in close agreement with previously published results (1), and were observed with other vegetative rods (13) and viruses (17). The detection limit observed with BG spores following processing with the hybrid method is in agreement with some studies (1, 3, 8), but higher than the 25 - 250 spores/ml reported in other studies (7, 18). It is important to note that these lower claims came from performing PCR directly from relatively inhibitor-free samples, and without a procedure for the removal of extraneous non-nucleic acid material from environmental samples. The study by Kuske *et al.* (15) employed the use of bead mill homogenization to effectively lyse *Bacillus* spores. Manual steps such as bead beating and sonication are undesirable to a completely automated approach, and have additional problems like the creation of aerosols of biological materials, and that require disruption facilitators such as silica or zirconium beads that have to be removed from each sample downstream in the process. In spite of its limitations, bead beating was shown to greatly increase the limit of detection of BG spores in our study and would likely be an essential part of biological agent detection. It is important to note that the detection limits in this study were determined using *Erwinia herbicola* and BG spore preps where every effort was made to remove all extracellular debris through a series of aggressive washing steps. It is likely that real-life environmental samples would have some vegetative growth, thus improving the detection limit when clumped and dirty spore preparations are extracted using this system (4).

The developed hybrid protocol was effective in removing PCR inhibitors from washes of air filters, surface swipes, and eight different soil types, as observed by the minimal shift in C_T values observed in spiked samples. The level of detection that our method obtains meets our internal requirements for environmental samples, and the DNA extraction step is accomplished in only 70 min. without requiring a spore germination step (14). In combination with an integrated automation system, the entire protocol is performed without user intervention, greatly reducing our staffing requirements, and thus reducing our overall cost.

The Automated Biological Agent Testing System (ABATS) has been designed to reduce the turn-around time for analysis of unknown environmental samples as part of a routine bio-surveillance operation or to serve as a replacement for manual laboratories in the event of sample

surge at high impact testing sites. As part of the labor reduction effort, we have developed a hybrid protocol for extraction of nucleic acids from environmental samples that can be fully automated and represents a significant increase in sample throughput compared to manual DNA preparation methods. The ABAT system can realize a two-thirds reduction in the cost per sample based upon our current operational capability in which 300 samples are processed per day for six bio-threat agents. Higher sample numbers that process 1,000 samples a day are possible and will reduce cost per sample even more using this system. The use of the magnetic bead resins is in their capacity for full automation, ability to handle real world environmental samples, and to isolate total nucleic acids. The use of this purification method on the Biomek® FX will be useful for screening sites in which smaller numbers of samples will be processed. This method is being used as part of a concept called Stations of Robotic Monitoring (STORM), which seeks to employ islands of automation to automate only the slowest steps in a testing process. Clearly, the linkage of automated processing systems with traditional laboratory methodologies has allowed for an increase in the efficiency of our operations and can provide a cost savings to national homeland defense efforts.

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